Original Article

EFFECT OF TIME BETWEEN CARBON DIOXIDE TREATMENTS ON THE ONSET OF OVIPOSITION IN QUEEN HONEY BEES

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Received: 11 April 2022; accepted: 14 November 2022

Abstract

It is commonly known that two carbon dioxide treatments accelerate the beginning of egg laying in honey bee queens. However, the minimum time between anaesthesias needed for ovary activation has not yet been examined. The aim of the study was to investigate the effect of time between carbon dioxide treatments on the onset of oviposition in instrumentally inseminated and virgin queen honey bees. About half of the queens were anaesthetised before insemination and a second time during insemination, and the rest of the queens were anaesthetised two times without insemination. The time between carbon dioxide treatments was as follows: 96, 48, 24, 12, 6, 3 hrs, 10 min, 5-6 sec. Queens in a control group were anaesthetised only once. The mean number of days between the second treatment and the onset of oviposition in particular groups and in the control group was 10.3, 7.6, 9.9, 10.5, 7.8, 10.9, 14.2, 15 and 21.9, respectively, and the median was 9, 7, 7, 8, 7, 8.5, 11.5, 13 and 21 days, respectively. Queens in groups 5-6 sec and 10 min began oviposition significantly earlier than those in control group, but significantly later than in groups from 3 to 96 hrs. Instrumentally inseminated queens started to lay eggs at the same time (mean 12 and median 9 days after second treatment) as queens treated with carbon dioxide without insemination (mean 11.7 and median 9 days).

Keywords: anaesthesia, carbon dioxide treatment, instrumental insemination, onset of oviposition, queen honey bee, virgin queen

INTRODUCTION

Many authors have studied factors affecting the onset of oviposition in instrumentally inseminated queen honey bees, but some of the results are contradictory. This is most often due to some other factor at work that escapes the attention of researchers. For example, Ebadi & Gary (1980) found a significant correlation between the number of spermatozoa in the spermatheca and the oviposition onset. The authors reported that queens which had 2.02, 3.05, 3.33, and 4.64 million (on average) spermatozoa started oviposition 16.3, 12.3, 10.0, and 5.6 days, respectively, after the insemination. On the contrary, Gąbka (2022) showed no significant differences in the beginning of egg laying in queens with less than 3 million or 3-4, 4-5, and more than 5 million spermatozoa in the spermathecae. Also, different results were obtained in the case of flights' effect on the onset of oviposition in instrumentally inseminated queens. Gerula et al. (2011) stated that the insemination of queens after their orientation flight does not affect the onset of oviposition. Ebadi & Gary (1980) demonstrated that instrumentally inseminated queens with no flight experience started laying eggs significantly earlier than those stimulated to fly before insemination. The flights lasted for at

least ten minutes before any drones were flying. According to Woyke et al. (2008), instrumentally inseminated queens, which had been flying for three minutes on a window inside a room before being inseminated, initiated oviposition significantly earlier than those without flight. The same flight after insemination, though, did not affect the onset of oviposition. In past research experiments, carbon dioxide exposure used for the anaesthesia of queens usually lasted for three minutes (Camargo & Goncalves, 1971; Engels et al., 1976; Woyke et al., 1995, 2008; Chuda-Mickiewicz et al., 2012; Gąbka, 2022) or ten minutes (Mackensen, 1947; Engels et al., 1976; Ebadi & Gary, 1980; Kaftanoglu & Peng, 1980, 1982; Moritz & Kühnert, 1984; Woyke & Jasiński, 1990; Manfredini et al., 2015). According to Konopacka (1991), onset of oviposition by instrumentally inseminated queen honey bees is directly related to the time of anaesthesia. The author reported a significantly shorter time between insemination and the beginning of egg laying after two ten-minute CO_2 treatments compared to one-minute treatments administered on two separate days. However, in one year, the results Konopacka obtained for the time between insemination and onset of oviposition were 8.10 and 22.15 days, respectively, and for the next year 7.25 and 12.38, respectively. The difference between the years in the case of the one-minute treatment was ten days, so another factor must have been involved. On the contrary, Gąbka (2019) obtained the same results after ten-minute and after one-minute treatments. Ebadi & Gary (1980) reported a significantly longer time from insemination to oviposition in queens that were narcotised once with 100% CO_2 compared to lower concentrations of carbon dioxide. The opposite results were obtained by Bieńkowska et al. (2012) who reported a shorter time through a single application of 100% CO_2 than an application of 75-90%. Chuda-Mickiewicz et al. (2012) showed that queens anaesthetised during insemination with 40% CO_2 in N_2, initiated oviposition significantly later than those treated with 60% CO_2 in N_2, and those treated with 100% CO_2. Gąbka (2019), however, stated no significant differences in the beginning of egg laying in queens anaesthetised with carbon dioxide or nitrogen (N_2). Konopacka (1991) noticed the same effect of N_2O (nitrous oxide) as CO_2, on the onset of oviposition.

The level of vitellogenin in queens increases after natural mating (Kaatz, 1984) and is higher than after instrumental insemination in which only one CO_2 treatment was used (Kocher et al. 2010). Two anaesthesias with CO_2 stimulate vitellogenin synthesis in unmated queens (Engels et al, 1976; Thompson et al., 2007). It does not occur after a single treatment (Niño et al., 2011, 2013). Two carbon dioxide treatments also induce ovary activation and accelerate egg laying in instrumentally inseminated honey bee queens (Mackensen, 1947; Ebadi & Gary, 1980; Konopacka, 1991; Woyke et al., 2001). However, it does not matter if the queens are inseminated because the same effect is obtained in virgins (Mackensen, 1947; Kaftanoglu & Peng, 1982; Harris et al., 1996; Thompson et al., 2007; Gąbka, 2019). There are no significant differences in the ovary activation of queens inseminated with semen or inseminated with only a saline solution (Niño et al., 2013). The expression of many genes in queens after two anaesthesias with CO_2 changes similarly to that seen in mated queens (Manfredini et al., 2015). It is commonly known that two carbon dioxide treatments accelerate the beginning of egg laying in queens. Additional anesthesia is most commonly used one or two days before or after insemination (Mackensen, 1947; Kaftanoglu & Peng, 1980, 1982; Harbo & Szabo, 1984; Moritz & Kühnert, 1984; Skowronek et al., 1995; Woyke et al., 2001, 2008; Chuda-Mickiewicz et al., 2012; Gerula et al., 2011, 2016; Manfredini et al., 2015; Gąbka, 2022). However, the minimum time between anaesthesias needed for ovary activation has not yet been examined.

The aim of the study was to investigate how the time between carbon dioxide treatments affects the onset of oviposition in queen honey bees. The experiment was carried out on instrumentally inseminated queens and on virgins to eliminate the influence of insemination and to confirm that not insemination but the

second anaesthesia is significant. The effect of storage conditions of queens between the two treatments on the onset of oviposition was also investigated.

MATERIAL AND METHODS

The study was conducted at the Warsaw University of Life Sciences, Poland. Two experiments were performed, first in 2019-2020 and second in 2021. A total of 227 Carniolan queens (*A. m. carnica*) were investigated, 204 in the first experiment and 23 in the second. Queen cells or one-day-old virgin queens were introduced into mating nuclei which contained about 1,000 workers. To prevent natural mating, queen excluders were placed at the entrances of the nuclei. All queens were marked by number tags so they could be identified and more easily be found in the colonies. In the first experiment, about half of the queens were anaesthetised with CO_2 before insemination and second time during insemination, and the rest of the queens were anaesthetised two times without insemination. Both queens inseminated and queens anaesthetised without insemination were divided into nine groups. These groups differed in time between first and second treatment: 96, 48, 24, 12, 6, 3 hours, 10 minutes, 5-6 seconds, and queens anaesthetised only once, as a control group. Queens were taken from their nuclei directly before treatment and placed back immediately after the treatment. The exceptions were queens from groups 5-6 sec and 10 min, which stayed in the laboratory in the time between the two treatments. Queens were anaesthetised for one minute each time. The second anaesthesia was at the age of eight days. In the second experiment, all queens were treated with CO_2 twice without insemination. The second treatment was performed one hour after the first. As in the first experiment, queens were treated for one minute at the age of eight days. They were divided into two groups; in the first, queens were put into their nuclei in time between the two treatments and in the second group they were kept without bees in incubator at temperature of 28°C. In both experiments, the nuclei were inspected daily or every other day for egg laying.

The Kolmogorov–Smirnov test was applied to determine whether the distribution of the onset of oviposition significantly differed from normal. For statistical comparisons, the Mann-Whitney test (for two groups) and the Kruskal-Wallis test (for more than two groups) were used. Both of these tests are nonparametric and compare the medians instead of the means. The differences in the mortality of queens instrumentally inseminated and queens anaesthetised without insemination were analysed using the χ^2 test.

RESULTS

Out of 103 instrumentally inseminated queens eight died and out of 101 virgins in the first experiment only one. The difference was statistically significant (χ^2 test: $p=0.018$). No queen died in the second experiment. Onset of oviposition did not have a normal distribution in both the first (Kolmogorov-Smirnov test: $p<0.001$) and the second experiment (Kolmogorov-Smirnov test: $p=0.028$). The time between the first and second CO_2 treatments affected significantly the commencement of egg-laying (Kruskal-Wallis test: $p<0.001$). Queens in groups 5-6 sec and 10 min began oviposition significantly earlier than in the control group, but significantly later than in groups from 3 to 96 hrs (Tab. 1). No significant differences were found in the onset of oviposition of queens instrumentally inseminated and queens anaesthetised without insemination (Mann-Whitney test: $p=0.898$) (Tab. 2). The storage conditions of queens in the time between two CO_2 treatments affected significantly the beginning of egg laying (Tab. 3). Queens kept for one hour without bees in an incubator at a temperature of 28°C started oviposition later than the queens that were in this time in their nuclei (Mann-Whitney test: $p=0.008$).

Table 1.

Onset of oviposition of queens depending on time between first and second CO_2 treatment (days after second treatment)

Time between treatments	Number of queens	Min - Max	Mean ± sd	Median
96 hrs	18	3 - 33	10.3 ± 7.84	9[a]
48 hrs	22	2 - 26	7.6 ± 5.05	7[a]
24 hrs	29	2 - 34	9.9 ± 7.65	7[a]
12 hrs	19	2 - 30	10.5 ± 8.60	8[a]
6 hrs	19	2 - 17	7.8 ± 4.47	7[a]
3 hrs	22	2 - 41	10.9 ± 9.48	8.5[a]
10 min*	30	4 - 35	14.2 ± 7.98	11.5[b]
5-6 sec*	19	3 - 37	15.0 ± 7.87	13[b]
Control**	17	6 - 41	21.9 ± 10.52	21[c]
Overall	195	2 - 41	11.9 ± 8.65	9

Different letters indicate significant differences between medians ($p<0.05$)
*Time counted from waking up after first anaesthesia
**Queens anaesthetised only once

Table 2.

Onset of oviposition by instrumentally inseminated queens and virgins (days after second CO_2 treatment)

Queens	Number of queens	Min - Max	Mean ± sd	Median
Inseminated	95	2 - 35	12.0 ± 8.87	9[a]
Virgins	100	2 - 41	11.7 ± 8.48	9[a]
Overall	195	2 - 41	11.9 ± 8.65	9

Same letters indicate no significant differences between medians ($p>0.05$)

Table 3.

Onset of oviposition by virgin queens depending on storage conditions in time between two CO_2 treatments (days after treatments)

Storage conditions*	Number of queens	Min - Max	Mean ± sd	Median
Nuclei	12	6 - 29	12.5 ± 7.34	10 a
Incubator	11	6 - 35	21.7 ± 9.35	17 b
Overall	23	6 - 35	16.9 ± 9.43	14

Different letters indicate significant differences between medians ($p<0.05$)
*Time of storage between treatments was 1 hour

DISCUSSION

The onset of oviposition in this experiment does not have a normal distribution for both instrumentally inseminated queens and virgins. Therefore, the median value is significant, not the mean. This confirms results obtained by Woyke et al. (2008), Gerula et al. (2011), Gąbka & Woyke (2014) and Gąbka (2022). Throughout the discussion, however, the number of days to oviposition refers to the mean value, because most authors compare in statistical analysis the mean, not the median.

The percent of dead queens after insemination in this experiment (7.8) was similar to the results reported by others (3.3-7.9) (Bieńkowska & Panasiuk, 2006; Chuda-Mickiewicz et al., 2012; Gąbka et al., 2016; Gąbka & Cobey, 2018; Gąbka, 2022). In the experiments by Ebadi & Gary (1980), Kaftanoglu & Peng (1980, 1982) and Moritz & Kühnert (1984), the mortality rates were higher (16-35%). There is always a risk of injury or infection during insemination. The confirmation of this is that only 1% out of queens anaesthetised without insemination died.

The results of our study showed that instrumentally inseminated queens start to lay eggs at the same time as queens treated with carbon dioxide without insemination. Other authors (Mackensen, 1947; Kaftanoglu & Peng, 1982; Harris et al., 1996; Thompson et al., 2007; Gąbka, 2019) also stated that CO_2 anaesthesia induces ovary activation and egg laying in virgin queens. For the commencement of oviposition, not the insemination but the second anaesthesia is significant. An additional carbon dioxide treatment is the main factor affecting the acceleration of oviposition onset in instrumentally inseminated queens (Mackensen, 1947; Ebadi & Gary, 1980; Konopacka, 1991; Woyke et al., 2001). This is confirmed by research on gene expression and changes in the level of vitellogenin (Engels et al, 1976; Thompson et al., 2007; Kocher et al. 2010; Niño et al., 2011, 2013; Manfredini et al., 2015), which plays a significant role in ovary development (Valle, 1993). In our experiment, queens anaesthetised or inseminated once started oviposition significantly later (21.9 days after treatment) than queens anaesthetised two times (with or without insemination) (from 7.6 to 10.9 days). As reported by others, the beginning of egg laying, after insemination, and with only one anaesthesia treatment during insemination, was (on average): 10-11 (Woyke et al., 2001), 17.6 (Bieńkowska et al., 2012), 18.3 (Gerula et al., 2011), 20.3-22.3 (Konopacka, 1991), 22.7 (Kahya & Gençer, 2022) and 28.3 days (Ebadi & Gary, 1980). Some of the results are similar to those obtained in this experiment but there is a large range between the above-mentioned mean number of days from insemination to oviposition. All these authors used the same semen dose (8 µl), so there were other factors at work. In the case of double insemination or single insemination with additional anaesthesia, the time from second treatment to oviposition, reported by others, was similar to that obtained in our study and ranged from 5.7 to 11 days (Camargo & Goncalves, 1971; Ebadi & Gary, 1980; Kaftanoglu & Peng, 1980, 1982; Moritz & Kühnert, 1984; Woyke & Jasiński, 1990; Wilde, 1994; Skowronek et al., 1995; Woyke et al., 2008; Gerula et al., 2011, 2016; Chuda-Mickiewicz et al., 2012; Gąbka, 2022).

Gąbka (2022) stated that the semen dose does not affect the commencement of egg-laying. On the contrary, Ebadi & Gary (1980) reported that queens inseminated with 16 µl began oviposition significantly earlier than those inseminated with 8 µl. However, the maximum amount that the syringe tip could hold, in that experiment, was only 10 µl. So, two consecutive injections of 8 µl each were made when queens were inseminated with 16 µl of semen. The authors described that the second injection was done after the syringe was re-loaded. Probably, the queens woke up after first insemination. If so, the earlier start of egg laying was due to the second anaesthesia resulting from second insemination. This could explain why the authors obtained completely different results than Gąbka (2022). The results of our experiment prove that even a very short time between two anaesthesias induces ovary activation. Queens treated with CO_2 after 5-6 sec or 10 min from waking up after the first

treatment started to lay eggs significantly earlier than queens anesthetised once but significantly later than those treated again after 3-96 hours from the first anaesthesia. Also, it turned out that storage conditions of queens between two CO_2 treatments were important. Queens kept in this time without bees in an incubator at a temperature of 28°C started to lay eggs significantly later than the queens that were in their nuclei. It is possible, however, that some other factor that is difficult to define was at work, which should be further investigated.

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